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published in

Infection and Immunity
2009

DOI (link to publisher)

[10.1128/IAI.01256-08](https://doi.org/10.1128/IAI.01256-08)

document version

Publisher's PDF, also known as Version of record

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citation for published version (APA)

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Role of Phosphatidylinositol Mannosides in the Interaction between Mycobacteria and DC-SIGN[†]

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Received 14 October 2008/Returned for modification 8 December 2008/Accepted 29 July 2009

The C-type lectin dendritic cell (DC)-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN) is the major receptor on DCs for mycobacteria of the *Mycobacterium tuberculosis* complex. Recently, we have shown that although the mannose caps of the mycobacterial surface glycolipid lipoarabinomannan (ManLAM) are essential for the binding to DC-SIGN, genetic removal of these caps did not diminish the interaction of whole mycobacteria with DC-SIGN and DCs. Here we investigated the role of the structurally related glycolipids phosphatidylinositol mannosides (PIMs) as possible ligands for DC-SIGN. In a binding assay with both synthetic and natural PIMs, DC-SIGN exhibited a high affinity for hexamannosylated PIM₆, which contains terminal $\alpha(1\rightarrow2)$ -linked mannosyl residues identical to the mannose cap on ManLAM, but not for di- and tetramannosylated PIM₂ and PIM₄, respectively. To determine the role of PIM₆ in the binding of whole mycobacteria to DC-SIGN, a mutant strain of *M. bovis* bacillus Calmette-Guérin deficient in the production of PIM₆ (Δ pimE) was created, as well as a double knockout deficient in the production of both PIM₆ and the mannose caps on LAM (Δ pimE Δ capA). Compared to the wild-type strain, both mutant strains bound similarly well to DC-SIGN and DCs. Furthermore, the wild-type and mutant strains induced comparable levels of interleukin-10 and interleukin-12p40 when used to stimulate DCs. Hence, we conclude that, like ManLAM, PIM₆ represents a bona fide DC-SIGN ligand but that other, as-yet-unknown, ligands dominate in the interaction between mycobacteria and DCs.

Tuberculosis (TB), caused by the bacterium *Mycobacterium tuberculosis*, is a major cause of death world wide and claims approximately 1.7 million lives each year (74). Currently, it is estimated that about one-third of the total world population is latently infected and this number continues to rise. Besides macrophages, which are the primary target for infection by mycobacteria (21), dendritic cells (DCs) are pivotal in determining the nature of the antimycobacterial response (15, 35, 38). Importantly, mycobacteria, including the vaccine strain *M. bovis* bacillus Calmette-Guérin (BCG), are able to suppress the immune response by interfering with the normal function of DCs (23, 73). Induction of the immunosuppressive cytokine interleukin-10 (IL-10) has a negative effect on the production of the proinflammatory cytokine IL-12 and inhibits a Th1-biased response which is necessary for mycobacterial clearance (14, 45). Responsible for at least part of this immune modulation is the binding of mycobacteria to the C-type lectin DC-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN [CD209]) (26, 69). This lectin has been shown

to be the major mycobacterial receptor expressed by DCs (6, 66). DC-SIGN signaling enhances the transcription of the IL-10 gene upon Toll-like receptor (TLR) signaling (30). In addition, the level of DC-SIGN expression on host cells has been related to susceptibility to and the pathogenicity of mycobacterial infections (7, 51, 70).

The cell wall components on mycobacteria that dominate the interaction of mycobacteria with DC-SIGN are currently unknown. Several ligands have been studied so far: the mannosylated 19-kDa and 45-kDa antigens, mannose-capped lipoarabinomannan (ManLAM), arabinomannan (AM), lipo-mannan (LM), and phosphatidylinositol mannosides (PIMs) (27, 46, 59, 67). The 19- and 45-kDa glycoproteins were shown to have an inhibitory effect on mycobacterial binding to DC-SIGN (59). However, *M. tuberculosis* mutants deficient in these glycoproteins bound DC-SIGN as well as the wild-type strain did (59). For a long time, mycobacterial binding to DC-SIGN was thought to be mediated by ManLAM. Several studies showed that ManLAM, in contrast to AraLAM, which is ManLAM devoid of mannose caps, strongly binds to DC-SIGN and induces the production of IL-10 (27, 46). Yet, these studies were all performed with purified compounds. Strikingly, mutation of *capA* (Rv1635c), i.e., the gene responsible for the mannose capping of LAM (16), did not attenuate *M. bovis* BCG in in vitro and in vivo infection models (5). In addition, there was no significant reduction in binding of the mutant strain to DC-SIGN, compared to that of the wild-type strain (5). Therefore, additional DC-SIGN ligands must be present.

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[†] Supplemental material for this article may be found at <http://iai.asm.org/>.

[‡] Published ahead of print on 3 August 2009.

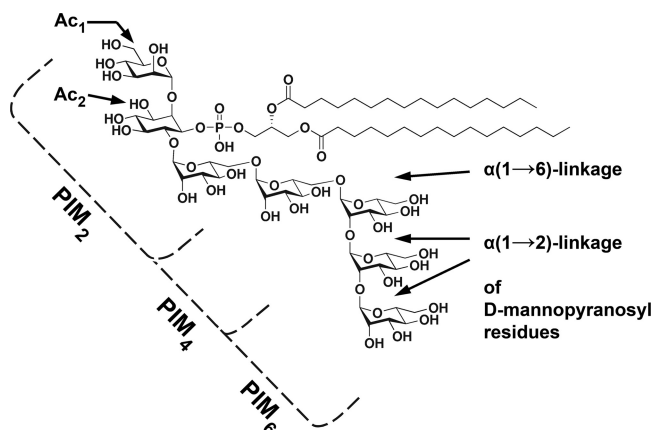


FIG. 1. Structure of synPIM₆. PIM₆ consist of a GPI anchor mannosylated with six mannosyl residues. natPIMs can be further acylated at the mannosyl group attached to the C-2 position of the inositol ring (Ac₁ PIMs) and subsequently at the C-3 position of inositol itself (Ac₂ PIMs). PIM₄ consists of a GPI anchor with four mannosyl residues in which the three mannosyl residues extending from the C-6 position of the inositol ring are linked in an $\alpha(1\rightarrow6)$ configuration. The terminal mannosyl residue in PIM₄ is substituted with two $\alpha(1\rightarrow2)$ -linked mannosyl residues to form PIM₆. This part of PIM₆ is identical to the $\alpha(1\rightarrow2)$ -linked mannosyl residues in the mannose cap on ManLAM.

The third group of ligands, the PIMs, were also shown to be able to bind to DC-SIGN (67). However, as for ManLAM, functional studies with live bacteria assessing the role of PIMs in the *Mycobacterium*–DC-SIGN interaction have yet to be conducted. PIMs are structurally related to LAM (for a review, see references 13 and 52). Like LAM, PIMs contain a mannosylated glycosylphosphatidylinositol (GPI) anchor, which can be further acylated at two positions (Fig. 1). The number of mannosyl residues can range from one to six, as is denoted by the subscript to PIM (PIM₁ to PIM₆), with tri- and tetra-acylated PIM₂ and PIM₆ as the predominant species (48). LAM and PIMs share the initial steps of their biosynthesis (40), with PIM₄ as the assumed branching point (11, 43). It is noteworthy that the $\alpha(1\rightarrow2)$ -linkage, which is only present between the terminal mannosyl residues of the polar PIMs, i.e., PIM₅ and PIM₆, is identical to the linkage between the terminal mannosyl residues in the mannose caps of LAM (Fig. 1). Previous studies have shown that DC-SIGN recognizes $\alpha(1\rightarrow2)$ -linked dimannosyl and trimannosyl structures, with the affinity increasing with length (20, 42, 61). Furthermore, PIMs are present in the outermost layer of the mycobacterial cell envelope (54), where they function as adhesins for mycobacterial binding to both macrophages and nonphagocytic cells (36, 47, 71). These observations suggest that PIMs may be involved in the interaction between mycobacteria and DC-SIGN.

In this study, we investigated the role of PIMs in the interaction between mycobacteria and DC-SIGN. We first determined the ability of DC-SIGN to interact with distinct species of PIMs by using both natural and synthetic PIMs (natPIMs and synPIMs, respectively). We found that DC-SIGN exhibited a high affinity for polar PIM₆, yet binding to the apolar, less mannosylated PIMs, i.e., PIM₂ and PIM₄, could not be observed. The next step was to further clarify the role of PIMs in

the binding of mycobacteria to DC-SIGN. The enzyme catalyzing the transfer of the fifth mannose to PIM₄ to produce PIM₅ is PimE (Rv1159) (49); thus, a mutant strain of *M. bovis* BCG (Δ pimE) that expresses PIM₂ and PIM₄ but not PIM₆ was created. To overcome the possibility of redundancy between ligands for DC-SIGN, we also constructed a double mutant devoid of both PIM₆ and the mannose caps on LAM (Δ pimE Δ capA). Unexpectedly, both mutant strains bound as well to both DC-SIGN and DCs as did the wild-type strain. Furthermore, when the wild-type and mutant strains were used to stimulate DCs, we did not find indications of a different skewing (IL-12p40/IL-10 ratio) of the immune response between the strains. Hence, we conclude that although PIM₆ is a ligand for DC-SIGN, its role in the interaction between mycobacteria and DC-SIGN, like that of the mannose caps on LAM, is limited. Binding of the mutant strains to DC-SIGN and DCs could still be blocked by mannan and antibodies against DC-SIGN, suggesting that other, as-yet-unknown, ligands for DC-SIGN must be present on the mycobacterial cell surface.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *M. bovis* BCG strain Copenhagen (8) was grown in Middlebrook 7H9 broth (Difco) with 10% Middlebrook albumin-dextrose-catalase enrichment (BBL) and 0.05% Tween 80 or on Middlebrook 7H10 agar (Difco) with 10% Middlebrook oleic acid-albumin-dextrose-catalase enrichment (BBL) at 37°C. *Escherichia coli* strain DH5 α (32) was grown on Luria-Bertani agar at 37°C. The concentrations of antibiotics used were 25 μ g ml⁻¹ kanamycin and 50 and 100 μ g ml⁻¹ hygromycin for *M. bovis* BCG and *E. coli*, respectively.

Construction of *M. bovis* BCG mutant strains. An unmarked deletion mutation in *pimE* (BCG_1220, homolog of Rv1159) was constructed in both wild-type *M. bovis* BCG and the *M. bovis* BCG Δ capA (BCG_1673c, homolog of Rv1635c) mutant, which produces LAM devoid of mannose caps (5), by using a two-step strategy involving vectors pGOAL19 and pNIL (57). First, two fragments harboring the up- and downstream regions of *pimE*, respectively, were amplified by PCR from *M. bovis* BCG genomic DNA with primer set BCG_1220-LF (5'-CTGGGCAACTATTGGTGGT-3') and BCG_1220-LR (5'-CAGGTGATGATCCGCTCTTT-3'), primer set BCG_1220-RF (5'-CGATCGAGGGGTACATGAG-3') and BCG_1220-RR (5'-CAGATAGGTCCAGGCGAGTC-3'), and *Pfu* DNA polymerase (Fermentas). The temperature program was as follows: 94°C for 5 min; 30 cycles of 30 s at 96°C, 30 s at 56.5°C, and 2 min at 72°C; followed by 7 min at 72°C and subsequent cooling to 4°C. PCR products were cloned separately into pCRII-TOPO according to the manufacturer's instructions (Invitrogen). They were cut out by using the *Eco*RI sites of pCRII-TOPO and ligated to each other in order to obtain the knockout *pimE* construct in which 771 bp of the gene were deleted. Constructs were checked for the correct orientation of the two fragments with primers BCG_1220-LR and BCG_1220-RF and re-cloned into pCRII-TOPO. A *Hin*DIII/*Pst*I digest from pCRII-TOPO- Δ pimE was ligated into *Hin*DIII/*Pst*I-digested pNIL. A *hyg*-*P_{Ag85}*-*lacZ*-*P_{hsp60}*-*sacB* marker was cassette cut out from the pGOAL19 plasmid with *Pac*I and subsequently cloned into the *Pac*I site of pNIL- Δ pimE to obtain the final plasmid, which was then electroporated into wild-type and Δ capA mutant *M. bovis* BCG (56). Selection of mutants was performed as described previously (57). In short, after single-crossover events, we selected Kan^r Hyg^r Suc^s blue colonies which harbor both wild-type *pimE* and a *pimE* gene with a deletion mutation, the hygromycin and kanamycin resistance genes (*hyg* and *kan*, respectively), *lacZ*, and *sacB*. Double-crossover events were induced by selection on plates containing 2% (wt/vol) sucrose. The colonies obtained (Kan^r Hyg^r Suc^s) either contained a wild-type or a mutant copy of *pimE*. Potential mutants were screened for the presence or absence of the deletion fragment by PCR with various primer sets.

Complementation of *M. bovis* BCG mutant strains. The *pimE* gene was amplified from genomic BCG DNA with primers BCG_1220-F-BamHI (5'-GGATCCGATGTTGGCATGTCTCT-3') and BCG_1220-R (5'-AGGTGGTATCA CGGAAAACG-3') and *Pfu* DNA polymerase (Fermentas). The following PCR program was used: 95°C for 3 min; 35 cycles of 30 s at 94°C, 30 s at 55°C, and 3 min at 72°C; and 7 min at 72°C. The PCR product obtained was cloned into pCRII-TOPO. This plasmid was digested with BamHI and *Eco*32I, and the

fragment was ligated into BamHI/Eco32I-digested pSMT3-eGFP (1, 33). In the resulting plasmid, pSMT3-*pimE*, the *pimE* gene was located behind the heat shock promoter 60 gene. pSMT3-*pimE* was isolated from *E. coli* DH5 α cells with a QIAprep Miniprep kit (Qiagen) and electroporated into *M. bovis* BCG Δ *pimE* as described previously (55). Transformants were selected on hygromycin. For complementation of the *M. bovis* BCG Δ *pimE* Δ *capA* mutant, *pimE* was amplified with primers BCG_1220-F-BamHI and BCG_1220-R-BclI (5'-ACTAGTA GGTGGTATCACGGAAAA-3') with the following PCR program: 95°C for 5 min; 35 cycles of 30 s at 94°C, 30 s at 58°C, and 3 min at 72°C; and 7 min at 72°C. The PCR product was cloned into pCRII-TOPO. The BamHI/BclI digest of this plasmid was cloned into the BamHI/BclI site of plasmid pSMT3-*capA* (5) to obtain pSMT3-*pimE-capA*.

Mabs and DC-SIGN-Fc. Murine monoclonal antibodies (MAbs) of the immunoglobulin M (IgM) class were used. MAb F30-5 recognizes the carbohydrate structure (Ara)₆ (for its structure, see reference 42) and binds both ManLAM and AraLAM (5, 41). MAb 55.92.1A1 binds the mannose cap on LAM (5). F183-24 recognizes α (1 \rightarrow 2)-linked mannosyl residues as present in the mannose cap on ManLAM and PIM₆ (41). The DC-SIGN-Fc construct consists of the extracellular portion of DC-SIGN C terminally fused to a human IgG1-Fc fragment (25). In all experiments involving DC-SIGN-Fc, Tris-buffered saline containing 20 mM Tris, 150 mM NaCl, 1 mM MgCl₂, 2 mM CaCl₂ (TSM), and 0.5% bovine serum albumin (Fluka) was used instead of phosphate-buffered saline (PBS).

PIMs. Synthetic 2,6-(di- α -D-mannopyranosyl)-1-*O*-(1,2-di-*O*-palmitoyl-*sn*-glycero-3-phosphoryl)-D-*myo*-inositol (synPIM₂) and synthetic 1-*O*-(1,2-di-*O*-hexadecanoyl-*sn*-glycero-3-phosphoryl)-2-*O*-(α -D-mannopyranosyl)-6-*O*-[(α -D-mannopyranosyl)-(1 \rightarrow 6)-(α -D-mannopyranosyl)-(1 \rightarrow 6)-(α -D-mannopyranosyl)]-D-*myo*-inositol (synPIM₄) were prepared by following methods published elsewhere (3). A synthetic sample of 1-*O*-(1,2-di-*O*-hexadecanoyl-*sn*-glycero-3-phosphoryl)-2-*O*-(α -D-mannopyranosyl)-6-*O*-[(α -D-mannopyranosyl)-(1 \rightarrow 2)-(α -D-mannopyranosyl)-(1 \rightarrow 2)-(α -D-mannopyranosyl)-(1 \rightarrow 6)-(α -D-mannopyranosyl)]-D-*myo*-inositol (synPIM₆) was synthesized from the starting material 1-*O*-allyl-3,4,5-tri-*O*-benzyl-D-*myo*-inositol. Key synthetic steps included the thioglycosylation of 1-*O*-allyl-2-*O*-(2-*O*-benzoyl-3,4,6-tri-*O*-benzyl- α -D-mannopyranosyl)-6-*O*-(2-*O*-benzoyl-3,4-di-*O*-benzyl- α -D-mannopyranosyl)-3,4,5-tri-*O*-benzyl-D-*myo*-inositol with phenyl (2-*O*-benzoyl-3,4,6-tri-*O*-benzyl- α -D-mannopyranosyl)-(1 \rightarrow 2)-(3,4,6-tri-*O*-benzyl- α -D-mannopyranosyl)-(1 \rightarrow 2)-(3,4,6-tri-*O*-benzyl- α -D-mannopyranosyl)-(1 \rightarrow 6)-2-*O*-benzoyl-3,4-di-*O*-benzyl-1-thio- α -D-mannopyranoside in 76% yield and introduction of the diacylglycerol moiety by phosphoramidite coupling methodology. synPIM₆ analyzed for C₇₇H₁₃₈O₄₃P; high-resolution mass spectrometry-electrospray ionization [M-H][−] found, 1,781.8358. synPIM₆ was further characterized by treatment with hydrazine to afford dPIM₆; ¹H NMR (500 MHz, D₂O) δ 5.30 (br s, 1H), 5.20 (br s, 1H), 5.13 (br s, 1H), 5.12 (br s, 1H), 5.06 (br s, 1H), 4.91 (br s, 1H), 4.35 (br s, 1H), 4.23 to 3.59 (m, 45H), 3.39 (t, *J* = 9.1 Hz, 1H). ¹³C NMR (125 MHz, D₂O, selected data) δ 103.1, 102.6, 102.2, 101.6, 100.5, 99.2. High-resolution mass spectrometry-electrospray ionization [M-H][−]; calculated for C₄₅H₇₈O₄₁P, 1,305.3756; found, 1,305.3738.

The natPIMs (natPIM_{1,2} and natPIM₆) were purified from *M. tuberculosis* H37Rv and contained a mixture of differently acylated PIMs. The natPIMs were a gift from Colorado State University.

SDS-PAGE and immunoblotting. *M. bovis* BCG was grown until mid to late log phase and disrupted with a Beadbeater (BioSpec). Supernatants were equalized for protein content, as quantified by the bicinchoninic acid protein assay kit according to the manufacturer's instructions (Pierce). Cell lysates were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in glycine buffer for immunostaining with MAb F30-5 or 55.92.1A1 or to 12% SDS-PAGE in Tricine buffer for incubation with MAb F183-24 or DC-SIGN-Fc (44). After transfer onto a polyvinylidene difluoride membrane (Millipore) (68), the membrane was incubated in blocking reagents (Boehringer), followed by incubation with the MAbs. After washing, incubation with peroxidase-labeled goat-anti-mouse IgM (American Qualex) and 0.5% normal goat serum followed. Membranes probed with DC-SIGN-Fc were blocked with 1% bovine serum albumin (Fluka), and goat anti-human IgG-peroxidase (Jackson Laboratories) in 0.5% normal goat serum was used as the secondary antibody (5, 6). Membranes were developed with a mixture of 4-chloro-1-naphthol (Bio-Rad) and 3,3'-diaminobenzidine 4HCl (Sigma-Aldrich).

DC-SIGN-Fc adhesion assay. synPIMs and natPIMs were used to coat enzyme-linked immunosorbent assay (ELISA) plates (Nunc 96-well PolySorp) by adding methanol-dissolved PIMs (1 or 10 μ g per well) and overnight evaporation to dryness at room temperature. Titration of DC-SIGN-Fc (starting from 1.25 μ g ml^{−1}) was performed with the protocol as described for immunoblotting with *o*-phenylenediamine dihydrochloride (Sigma-Aldrich) as the coloring reagent

(27). To verify proper coating, ELISA plates were coated with various concentrations of PIMs and probed with MAb HA-1A, which recognizes hydrophobic structures (34). HA-1A bound to the less mannosylated PIMs as well as to PIM₆. The median staining ratios (measured by absorption at 490 nm) in nine data sets derived from three independent experiments were 0.9 for synPIM₂/synPIM₆, 1.1 for synPIM₄/synPIM₆, and 0.9 for natPIM_{1,2}/natPIM₆.

Two-dimensional (2D) thin-layer chromatography (TLC) for polar lipid analysis. Cells were harvested by centrifugation and washed once with PBS, and small-scale lipid extraction was performed to afford polar lipids as described previously (5, 10). The extracts were resuspended in CHCl₃-CH₃OH (2:1), and crude lipids were applied to the corners of aluminum-backed TLC plates (Merck). The plates were developed in solvent system E for polar lipid extracts (10). TLC was run once in each direction with CHCl₃-CH₃OH-H₂O (60:30:6) in the first direction and CHCl₃-acetic acid-CH₃OH-H₂O (40:25:3:6) in the second. Lipids were visualized by staining with α -naphthol and brief charring at 100°C until lipids appeared, which could then be compared to known standards (10).

Cells. Human peripheral blood mononuclear cells were isolated from buffy coats (Sanquin Blood Bank, Amsterdam, The Netherlands) by centrifugation on a Ficoll-Paque gradient (GE Healthcare), followed by selection for CD14⁺ monocytes with anti-CD14 MAb-coupled magnetic beads and the Midi-MACS system (Miltenyi Biotec) (30). Fresh monocytes were cultured in the presence of IL-4 (500 U ml^{−1}) and granulocyte-macrophage colony-stimulating factor (800 U ml^{−1}; PeproTech) to produce immature monocyte-derived DCs (MoDCs) (62), which were used for further experiments on day 6 after isolation. MoDCs, Raji cells, and transfected Raji cells expressing DC-SIGN (Raji + DC-SIGN cells) (24, 75) were cultured in RPMI 1640 medium (Gibco) supplemented with 10% fetal calf serum (Gibco), 100 U ml^{−1} penicillin, and 100 μ g ml^{−1} streptomycin at 37°C in a 5% CO₂ atmosphere.

Binding of DC-SIGN to PIMs and *M. bovis* BCG as determined by flow cytometry. synPIMs and natPIMs were used to coat 1.0- μ m fluorescent beads (Polysciences no. 17154) by adding 50 μ g PIMs to 1 ml 0.1 M carbonate-bicarbonate buffer (pH 9.6) containing 1.4×10^9 beads in presiliconized tubes (Sigma-Aldrich). After 1 h of rotating at 37°C, beads were collected by centrifugation and incubated for another hour in 1 ml 5% human serum albumin (HSA; Sigma-Aldrich) in TSM and washed in 0.5% HSA in TSM (65).

M. bovis BCG cultures were grown until mid-log phase, concentrated, and incubated with 1 mg ml^{−1} fluorescein isothiocyanate (Sigma-Aldrich) for 15 min at room temperature, after which the suspensions were washed and declumped by passage through a 5- μ m filter (Millipore).

Fluorescence of the different bead and mycobacterial suspensions was quantified with a FLUOstar-Galaxy microplate reader (BMG). Beads were added to human cells at a ratio of 50:1, and mycobacteria were added at a multiplicity of infection (MOI) of 0.5, 2, or 8. After incubation for 45 min at 37°C in the absence or presence of 2 mg ml^{−1} mannan (Sigma-Aldrich), the percentage of fluorescent cells was determined with a FACScan analytic flow cytometer (Becton Dickinson) and analyzed with the manufacturer's software (CellQuest version 3.1f).

Phagocytosis. MoDCs and mycobacteria were cocultured at a MOI of 1 to 2 (in triplicate in each independent experiment) for 3 h at 37°C, after which amikacin (Sigma) was added to a concentration of 200 μ g ml^{−1} for 2 h of incubation at 37°C. The MoDCs were then washed three times in PBS and finally lysed with 1% Triton X-100 (Sigma) in PBS for 20 min. Serial dilutions of the inoculum and the released mycobacteria were made in PBS-0.05% Tween 80 and plated on 7H10 agar. CFU were counted after 4 weeks (1).

Cytokine induction. MoDCs and mycobacteria were cocultured for 24 h at 37°C in the absence or presence of 10 ng ml^{−1} lipopolysaccharide (LPS) from *Salmonella enterica* serotype Abortusequi (Sigma-Aldrich). IL-10 and IL-12p40 concentrations in supernatants were analyzed with an ELISA against human IL-10 and IL-12p40 according to the manufacturer's instructions (BioSource).

Statistical analysis. All values were expressed as means of triplicates \pm standard deviation. The unpaired Student *t* test was used to identify differences between groups. A *P* value of <0.05 was considered statistically significant.

RESULTS

DC-SIGN has a higher affinity for PIM₆ than for less mannosylated PIMs. DC-SIGN exhibits a high affinity for α (1 \rightarrow 2)-linked mannosyl residues (42). As α (1 \rightarrow 2)-linked mannosyl residues are present in polar PIMs, i.e., PIM₆, but absent in apolar PIMs, i.e., PIM₄, PIM₂, and PIM₁ (Fig. 1), we set out to investigate the affinity of DC-SIGN for different species of PIMs with two independent assays. First, individual PIM com-

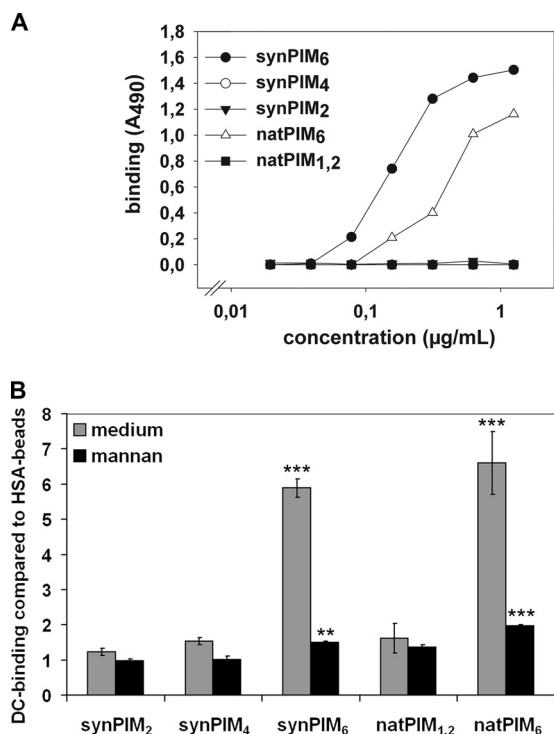


FIG. 2. Affinity of DC-SIGN(-Fc) for different species of PIMs (the degree of mannosylation is indicated by subscripts). (A) PIMs were used to coat ELISA plates; this was followed by titration with DC-SIGN-Fc. One representative experiment out of five (synPIMs) or three (natPIMs) is shown. (B) PIMs were used to coat fluorescent beads and analyzed for binding to MoDCs by flow cytometry. Indicated are the means of triplicates and the standard deviations. One representative experiment out of three (synPIMs) or two (natPIMs) is shown. This experiment was also repeated five and two times for the synPIMs and natPIMs, respectively, with Raji + DC-SIGN cells (see Fig. S1 in the supplemental material). ***, $P < 0.0005$; **, $P < 0.005$; *, $P < 0.05$ (compared to control HSA-beads).

pounds (purified natPIMs and synPIMs) were used to coat ELISA plates and probed with a DC-SIGN-Fc construct. As shown in Fig. 2A, DC-SIGN-Fc strongly bound to both synPIM₆ and natPIM₆ but not to synPIM₂, synPIM₄, and natPIM_{1,2} (a mixture of PIM₁ and PIM₂), suggesting that DC-SIGN-Fc specifically recognized PIM₆. To determine whether native, membrane-localized DC-SIGN exhibited a similar specificity, fluorescent polystyrene beads were coated with the

different species of PIMs and assayed for the capacity to bind to MoDCs (Fig. 2B) and Raji cells expressing DC-SIGN (see Fig. S1 in the supplemental material). Consistent with the results for DC-SIGN-Fc, only beads coated with PIM₆ (both natural and synthetic), compared to control beads coated with HSA, showed significantly increased binding. The interaction could be blocked by the addition of mannan (Fig. 2B) and by DC-SIGN-blocking antibody AZN-D1 (see Fig. S2 in the supplemental material), confirming that binding was dependent on DC-SIGN.

Construction of an *M. bovis* BCG *pimE* mutant deficient in polar PIM production. As demonstrated above, DC-SIGN specifically bound to PIM₆. To study the role of PIM₆ in the interaction between DC-SIGN and whole mycobacteria, our next goal was to create a mutant strain deficient in the production of PIM₆. Recently, it was demonstrated that *pimE* encodes the mannosyltransferase responsible for the elongation of PIM₄ to PIM₅ and that mutation of the gene abolished the production of polar PIMs (49). Hence, we created a *pimE* knockout of *M. bovis* BCG. As PIM₆ may show redundancy with ManLAM in binding to DC-SIGN, we also constructed a double knockout deficient in both PIM₆ and the mannose caps on LAM ($\Delta pimE \Delta capA$). First, the composition of PIMs in the cell wall of the wild-type and mutant strains was analyzed by 2D TLC. As shown in Fig. 3, all of the strains produced PIM₂, yet PIM₆ was only present in the wild-type strain. In contrast, the mutant strains accumulated PIM₄, which is consistent with the report on the mutation of the homologous gene in *M. smegmatis* mc²155 (49). Next, whole-cell lysates were probed by SDS-PAGE and immunoblotting with MAb F183-24, which recognizes PIM₆ (A. H. J. Kolk, unpublished data) (Fig. 4, lower left panel). In the low-molecular-weight part of the blot (<14 kDa), which is the region harboring the PIMs (39, 59), F183-24 bound to PIMs from wild-type *M. bovis* BCG and the complemented strains (Fig. 4, lanes 1 and 6 and lanes 3 and 5, respectively) but not to PIMs from the $\Delta pimE$ and $\Delta pimE \Delta capA$ mutant strains (lanes 2 and 4, respectively). As expected, since the LAM/PIM biosynthesis pathway bifurcates after PIM₄ (11, 43), the mutant strains still produced LAM, as indicated by the retained interaction with anti-LAM MAb F30.5 (Fig. 4, upper left panel). *M. bovis* BCG $\Delta pimE \Delta capA$ only produced capless AraLAM, as shown by the lack of staining with cap-specific MAb 55.92.1A1 (Fig. 4, upper right panel, lane 4). Compared to wild-type *M. bovis* BCG, no additional

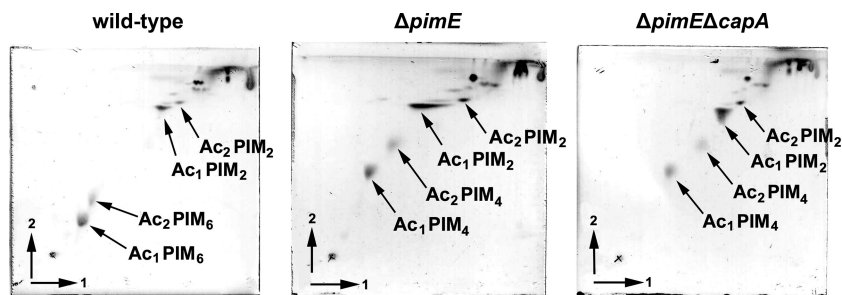


FIG. 3. 2D TLC lipid profile of wild-type and $\Delta pimE$ and $\Delta pimE \Delta capA$ mutant *M. bovis* BCG with solvent system E for polar lipid extracts (10). Ac₁ PIM₂, Ac₁ PIM₄, and Ac₁ PIM₆ are triacylated PIM₂, PIM₄, and PIM₆, respectively; Ac₂ PIM₂, Ac₂ PIM₄, and Ac₂ PIM₆ are tetra-acylated PIM₂, PIM₄, and PIM₆, respectively.

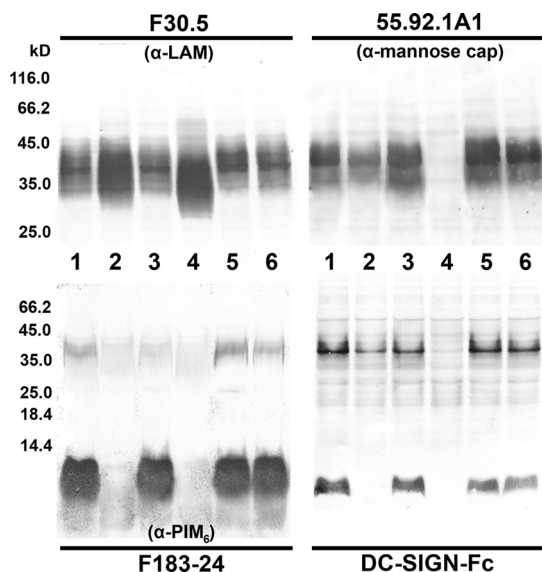


FIG. 4. SDS-PAGE and immunoblotting. *M. bovis* BCG cell lysates were subjected to glycine-SDS-PAGE (upper panels) or Tricine-SDS-PAGE (lower panels), transferred to polyvinylidene difluoride membrane, and probed with MAbs F30-5 (anti-AraLAM), 55.92.1A1 (anti-ManLAM), and F183-24 (anti-PIM₆) and DC-SIGN-Fc. The position of LAM on the blot is between 35 and 45 kDa, and that of the PIMs is below 14 kDa. Lanes 1 and 6, wild-type *M. bovis* BCG; lane 2, *M. bovis* BCG Δ *pimE*; lane 3, *M. bovis* BCG Δ *pimE* complemented with pSMT3-*pimE*; lane 4, *M. bovis* BCG Δ *pimE* Δ *capA*; lane 5, *M. bovis* BCG Δ *pimE* Δ *capA* complemented with pSMT3-*pimE-capA*.

alterations of the lipid composition in the mutant strains were observed (see Fig. S7 in the supplemental material).

Binding of DC-SIGN-Fc to LAM and PIMs from wild-type *M. bovis* BCG and the Δ *pimE* and Δ *pimE* Δ *capA* mutant strains. To evaluate the consequences of the *pimE* and *capA* mutations for the recognition of LAM and PIMs by DC-SIGN-Fc, whole-cell lysates from the wild-type and mutant strains were analyzed by Tricine-SDS-PAGE, followed by immunoblotting with DC-SIGN-Fc. As shown in Fig. 4 (lower right panel), DC-SIGN-Fc did not show binding at the position of PIMs for both the Δ *pimE* and Δ *pimE* Δ *capA* mutant strains (lanes 2 and 4, respectively). This binding pattern is similar to the immunostaining with F183-24 (Fig. 4, lower left panel, lanes 2 and 4, respectively), which confirms the selective affinity of DC-SIGN-Fc for α (1 \rightarrow 2)-interlinked mannosyl residues present in PIM₆. Loss of DC-SIGN-Fc binding to LAM without the mannose cap (Δ *capA*) has been demonstrated in previous studies (5, 27) and was also observed at the position of LAM in the cell lysate from *M. bovis* BCG Δ *pimE* Δ *capA* (Fig. 4, lane 4). Hence, in the *M. bovis* BCG Δ *pimE* Δ *capA* mutant, two ligands for DC-SIGN were absent. Complementation of the mutant strains restored the binding of DC-SIGN-Fc to both LAM and the PIMs (Fig. 4, lanes 3 and 5, respectively).

Binding to DC-SIGN and phagocytic uptake of wild-type *M. bovis* BCG and the Δ *pimE* and Δ *pimE* Δ *capA* mutant strains. To determine the effect of the *pimE* and *capA* mutations on the binding of whole mycobacteria to DC-SIGN-expressing cells, the wild-type and mutant strains were tested for the ability to bind MoDCs by flow cytometry. As shown in Fig. 5A, all three strains bound similarly well at all three of the MOIs tested. To

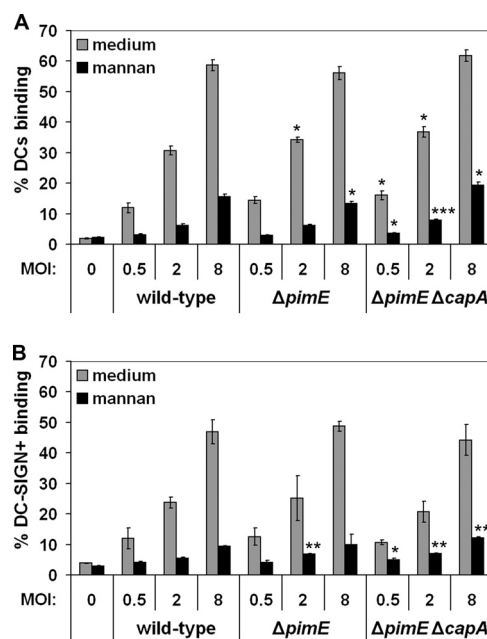


FIG. 5. Binding assay with (A) MoDCs and (B) Raji cells expressing DC-SIGN. Cells were incubated with wild-type *M. bovis* BCG or with the Δ *pimE* and Δ *pimE* Δ *capA* mutants at a MOI of 0.5, 2, or 8 for 45 min in the presence or absence of mannan. The percentage of the cell population binding *M. bovis* BCG was determined by flow cytometry. Shown are means of triplicates and the standard deviations. For binding to both MoDCs and Raji + DC-SIGN cells, one representative experiment out of three is shown. See Fig. S3 in the supplemental material for the control binding assay with wild-type Raji cells. ***, $P < 0.0005$; **, $P < 0.005$; *, $P < 0.05$ (compared to wild-type *M. bovis* BCG at equal MOIs).

test if a reduction in binding to DC-SIGN was possibly compensated for by other receptors on the MoDCs, the same assay was performed with Raji cells expressing DC-SIGN. As indicated in Fig. 5B, the *M. bovis* BCG Δ *pimE* and Δ *pimE* Δ *capA* mutants showed a cell-binding pattern similar to that of the wild-type strain. Furthermore, the binding to MoDCs could be blocked by the addition of mannan (Fig. 5A) and by DC-SIGN-blocking antibody AZN-D1 (see Fig. S4 in the supplemental material), confirming that the interaction of the mutant strains with MoDCs was still dependent on DC-SIGN. Although variation in binding was observed in some experiments, binding of the mutant strains was never significantly lower. In contrast, in three out of six experiments, a 1.5-fold increase in the binding of the mutant strains could be observed (data not shown). These data, together with the observation that the interactions could still be blocked by mannan, suggest that even in the Δ *pimE* Δ *capA* double knockout, additional ligands for DC-SIGN were present. Next, the wild-type and mutant strains were assayed for phagocytic uptake by MoDCs after 3 h of coculturing (see Fig. S5 in the supplemental material). A high variation in uptake efficiency was observed between the different donors. Nevertheless, the *M. bovis* BCG Δ *pimE* Δ *capA* mutant strain showed a consistent increase in phagocytic uptake compared to that of the wild-type strain in five of the six experiments (see Fig. S5 in the supplemental material). The median of the ratios of the uptake of *M. bovis* BCG Δ *pimE*

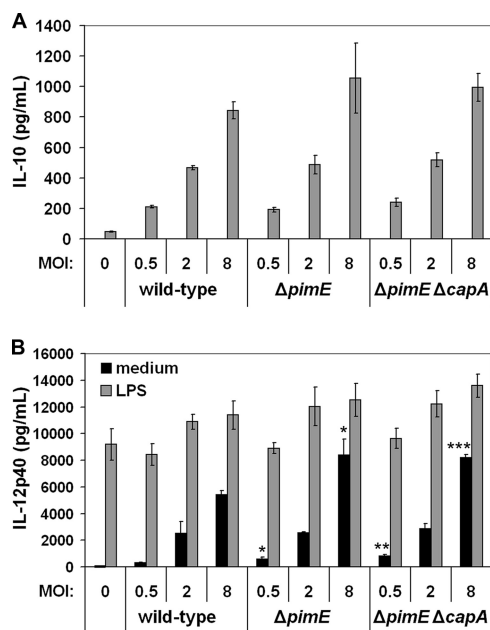


FIG. 6. Induction of IL-10 (A) and IL-12p40 (B) secretion in MoDCs by *M. bovis* BCG. MoDCs were cocultured with mycobacteria for 24 h in the absence or presence of LPS, after which cytokine secretion was measured by ELISA. Shown are means of triplicates and the standard deviations of one representative experiment (five were conducted). IL-10 secretion by non-LPS-activated MoDCs upon infection with BCG was below the limit of detection ($<20 \text{ pg ml}^{-1}$). ***, $P < 0.0005$; **, $P < 0.005$; *, $P < 0.05$ (compared to wild-type *M. bovis* BCG at equal MOIs).

$\Delta capA$ to the uptake of wild-type *M. bovis* BCG was 2.2, which is in line with the increased binding of the mutant strain that was observed in the earlier binding studies. The data from the CFU count of the *M. bovis* BCG $\Delta pimE$ mutant strain were less consistent. For two donors, the bacterial uptake was increased, whereas for the third donor it was lower (see Fig. S5 in the supplemental material). Hence, drawing conclusions for this mutant strain was not possible.

Wild-type *M. bovis* BCG and the $\Delta pimE$ and $\Delta pimE \Delta capA$ mutant strains induce similar cytokine profiles in MoDCs. DC-SIGN ligation interferes with TLR signaling and enhances transcription of IL-10 (27, 30). In addition, it has been shown that purified ManLAM inhibits the production of IL-12 by human DCs (53). Hence, removal of DC-SIGN ligands may induce an altered cytokine secretion profile in *Mycobacterium*-stimulated DCs. To determine the effect of the *pimE* and *capA* mutations on the ability to induce cytokine secretion in MoDCs, MoDCs (both immature and LPS primed) were stimulated with the wild-type and mutant bacteria, after which the amounts of secreted IL-10 and IL-12p40 were determined. Although the *Mycobacterium*-induced cytokine secretion varied among the five different donors tested, no consistent differences within a single donor were observed. Nevertheless, in some experiments, such as the one shown in Fig. 6, significant differences at certain MOIs could be observed. In general, the mutant strains induced more IL-12p40 and IL-10 (see Fig. S9 in the supplemental material). Of note, in all five of the donors tested, both immature and LPS-primed MoDCs produced IL-

12p40 following stimulation. In contrast, IL-10 secretion was only observed in LPS-primed MoDCs. Since bioactive IL-12 consists of two subunits, i.e., IL-12p40 and IL-12p35, with the p40 subunit also being present in other IL-12 family members, we also determined the amount of secreted IL-12p70. Although a difference in absolute cytokine production was observed, with the amount of secreted IL-12p40 being higher than that of IL-12p70 by a factor 10 to 100, depending on the donor, their relative levels of induction were similar (see Fig. S8 in the supplemental material). The ratios of IL-12p40/IL-10 induced by *M. bovis* BCG $\Delta pimE$ to IL-12p40/IL-10 induced by wild-type *M. bovis* BCG and of IL-12p40/IL-10 induced by *M. bovis* BCG $\Delta pimE \Delta capA$ to IL-12p40/IL-10 induced by wild-type *M. bovis* BCG were calculated for LPS-primed MoDCs from four different donors (one donor did not produce IL-10). These ratios ranged from 0.66 to 1.33 (22 triplicate data sets), with a median of 1.01. Hence, we concluded that the IL-12p40/IL-10 ratios of the two mutant strains were identical to that of the wild-type strain, meaning that the absence of PIM₆, either alone or in combination with missing mannose caps on LAM, did not alter the IL-12p40/IL-10 balance.

DISCUSSION

In this study, we demonstrated that the degree of mannosylation, in particular, the presence of $\alpha(1 \rightarrow 2)$ -linked mannosyl residues in polar PIM₆ (Fig. 1), determines the interaction of PIMs with DC-SIGN. DC-SIGN showed a high affinity for PIM₆ but not for less mannosylated PIMs, including PIM₄ (Fig. 2). Yet, a mutant strain deficient in the production of polar PIMs bound as well to DC-SIGN as did wild-type *M. bovis* BCG (Fig. 5). Even a double mutant which was also devoid of mannose caps on LAM did not bind less to DC-SIGN and DCs. Binding of the mutant strains could still be blocked by mannan, indicating that additional DC-SIGN ligands were present.

It has been suggested that mycobacteria target DC-SIGN to escape immune surveillance (26, 69). However, studies investigating single nucleotide polymorphisms in the promoter region of DC-SIGN have shown a reduced risk of TB associated with both downregulation (70) and upregulation (7) of DC-SIGN expression or even no association with susceptibility to TB at all (9, 29). A study with mice lacking SIGNR1—one of the murine homologs of DC-SIGN—showed that these mice had stronger T-helper 1 responses to *M. tuberculosis* and a reduced level of IL-10 (72). However, no differences in bacterial load and mouse survival rates could be detected (72). In a recent publication on human DC-SIGN transgenic mice, a decreased pathology and prolonged survival during mycobacterial infection have been reported. In this case, no differences in IL-10 secretion could be detected (64). It should be taken into account that the murine DC-SIGN system is different from the human one in both expression and specificity and it is not known yet which homolog is functionally most similar to the human DC-SIGN version (58, 60). Hence, many questions regarding the functional consequences of the interaction between mycobacteria and DC-SIGN still exist (51).

Mycobacterial strains that show a reduced binding to DC-SIGN will help to elucidate the role of DC-SIGN in mycobac-

terial pathogenesis. In principle, such strains can be developed by deleting the major DC-SIGN ligand(s) present. In contrast to what has long been thought, deletion of the mannose cap on LAM did not abrogate the DC-SIGN–mycobacterial interaction (5). Polar PIMs, i.e., PIM₅ and PIM₆, have been proposed before as possible ligands for DC-SIGN (67). Although it was shown that the degree of PIM mannosylation did not determine its binding to DC-SIGN (67), we decided to reconsider the case for two separate reasons. First, DC-SIGN shows an unambiguous high affinity of for $\alpha(1\rightarrow2)$ -interlinked mannosyl residues and this affinity increases with the number of mannosyl residues present, suggesting that DC-SIGN, in theory, should display an increased affinity toward higher-order PIMs (20, 42), and second, previous studies on PIMs were only performed with purified or synthetic compounds and not with whole mycobacteria.

In two independent assay systems with (i) ELISA plates coated with different species of PIMs and probing with DC-SIGN-Fc and (ii) PIM-coated beads used in binding studies with DC-SIGN expressing cells, we observed a clear difference in the affinity of DC-SIGN for the individual species of PIMs (Fig. 2); only PIM₆ was recognized by DC-SIGN. Even at high concentrations (10 μ g per well with ELISA) or at high bead-to-cell ratios (100:1; data not shown), we did not detect significant binding of DC-SIGN to synPIM₂, synPIM₄, or natPIM_{1,2}. Whether the degree and nature of acylation of the PIMs played a role in their binding to DC-SIGN was not directly assessed. However, these data suggest that this is not important because the synPIMs, which were all diacylated with identical fatty acids and were single discrete species, displayed binding properties similar to those of the analogous natPIMs, which contained mixtures of mono- to tetra-acylated PIMs. Moreover, natPIM₆ and synPIM₆ both showed a high affinity for DC-SIGN, indicating that differences in affinity are directly related to the degree of mannosylation. Strikingly, our results are different from those reported by Torrelles et al. (67). In that study, it was demonstrated that all PIMs, irrespective of their degree of mannosylation, showed similar binding to DC-SIGN. We have no ready explanation for the difference in outcome; however, we do note that the magnitude of the binding of PIMs to DC-SIGN reported in the study by Torrelles et al. was very small for all of the species tested and thus lacked specificity. Our data are more in agreement with a recent study by Boonyarattanakalin and coworkers (12). This study on the chemical synthesis of PIMs showed a high affinity of DC-SIGN for the polar PIMs, i.e., PIM₅ and PIM₆, and low or no affinity for the less mannosylated PIMs, i.e., PIM₂ and PIM₄, respectively, in a fluorescence scanning assay of a PIM microarray.

The *M. bovis* BCG Δ pimE and Δ pimE Δ capA mutant strains were created to investigate the role of polar PIMs in the interaction between DC-SIGN and whole mycobacteria. Although polar PIMs have been reported to be essential for cell wall integrity and mycobacterial growth (31), no differences in growth between the mutant and parent strains were observed (see Fig. S6 in the supplemental material), except for a higher tendency of the mutant strains to clump in older cultures (>1.5 weeks) at high optical densities at 600 nm (data not shown). Interestingly, the *M. bovis* BCG Δ pimE and Δ pimE Δ capA mutant strains accumulated PIM₄, a feature which was not observed in wild-type *M. bovis* BCG. This confirms that in

wild-type *M. bovis* BCG, PIM₄ is an intermediate in polar PIM biosynthesis (28).

The *M. bovis* BCG Δ pimE and Δ pimE Δ capA mutant strains did not show apparent phenotypic changes other than the altered production of PIM₆ and the mannose caps on LAM. Since DC-SIGN specifically bound PIM₆ in vitro (Fig. 2), we hypothesized that deletion of PIM₆ may reduce mycobacterial binding to cells expressing DC-SIGN. To test this, we first compared the wild-type and mutant bacteria for binding to MoDCs. The results indicated that both the *M. bovis* BCG Δ pimE and Δ pimE Δ capA mutant strains bound as well to MoDCs as did the wild-type strain (Fig. 5A). To exclude the possibility that other receptors on MoDCs masked a reduced affinity of DC-SIGN for the mutant strains, binding of the wild-type and mutant strains to Raji cells expressing DC-SIGN was also assessed. Also in this case, no reduction and in some cases even a small increase in the binding of the mutant strains compared to that of the wild-type strain was observed (Fig. 5B). This latter finding could be due to a less hydrophobic cell surface of the mutant strains, as was reported for an *M. smegmatis* Δ pimE mutant (49), since the increased binding was also observed for wild-type Raji cells, which do not express DC-SIGN (see Fig. S3 in the supplemental material). Binding of the mutant strains to MoDCs could still be blocked by mannan (Fig. 5), as well as by DC-SIGN-blocking antibody AZN-D1 (see Fig. S4 in the supplemental material). Therefore, we conclude that the absence of PIM₅ and PIM₆ in the mutant strains did not alter receptor usage for MoDCs: DC-SIGN was still the major receptor.

Finally, wild-type *M. bovis* BCG and the Δ pimE and Δ pimE Δ capA mutant strains were assayed for the ability to induce IL-12p40 and IL-10 secretion by MoDCs. The balance between IL-10 and IL-12 is important for shaping the host immune response, and alterations in it may thus influence the outcome of mycobacterial infections (4, 37, 50). Binding to DC-SIGN enhances the transcription of the IL-10 gene upon TLR signaling (30). Furthermore, PIMs have been reported to suppress allergic airway disease, which is associated with increased production of IL-10 (2, 63). However, PIM₁ and PIM₂ were used in the latter two studies (2, 63) and IL-10 induction in this case likely involves a different pathway (22). In our experiments, in which LPS-primed MoDCs were cocultured with wild-type *M. bovis* BCG or the Δ pimE and Δ pimE Δ capA mutant strains, no decrease in IL-10 secretion by MoDCs incubated with the mutant strains compared to wild-type *M. bovis* BCG was found (Fig. 6A). Instead, there was a tendency toward higher cytokine (IL-10, IL-12p40, and IL-12p70) induction by the mutant strains compared to that by wild-type *M. bovis* BCG (see Fig. S8 and S9 in the supplemental material). However, because the mutant strains induced higher production of both anti-inflammatory IL-10 and proinflammatory IL-12p40, the increased cytokine production is probably not biologically significant. Importantly, it should be noted that IL-10 induction by mycobacteria alone could not be detected. Only after costimulation with LPS did *M. bovis* BCG induce the secretion of IL-10. Since mycobacteria do not contain LPS, although other TLR4 ligands may be present (18), it will be important to investigate DC-SIGN-dependent IL-10 induction also with other classes of TLR ligands (particularly TLR2).

In summary, we have shown that although DC-SIGN has a

high affinity for PIM₆, this glycolipid does not dictate the *Mycobacterium*–DC-SIGN interaction. This finding demonstrates, like our previous study (5), that the interaction of mycobacteria with DC-SIGN is much more complex than initially anticipated. Furthermore, it demonstrates that studies with purified compounds should be interpreted with great care. In addition, our study shows that mycobacteria do not express one or two but probably many more ligands for DC-SIGN. As shown in Fig. 4 (lower right panel), DC-SIGN-Fc recognized, besides ManLAM and PIM₆, many additional bands (lane 4). These bands most likely represent mannosylated proteins, which are known to be expressed by mycobacteria (19). For at least the 45-kDa glycoprotein, it has been reported that the mannobiose and mannotriose units present are all $\alpha(1\rightarrow2)$ linked and thus reminiscent of the mannose cap on ManLAM (17). If these proteins are indeed involved in the binding of mycobacteria to DC-SIGN, creation of mutant strains displaying reduced binding will be a huge task to perform. It will therefore be of great importance to evaluate the role of mannosylated proteins in the binding of mycobacteria to DC-SIGN.

ACKNOWLEDGMENTS

B.J.A. acknowledges support for R.U. and J.G. from the EU (FP6 grant ImmunovacTB 37388). The mycobacterial glycolipids (natPIMs) were a gift from J. Belisle, Colorado State University, Fort Collins, and the National Institutes of Health, Bethesda, MD (contract NO1 AI-75320).

We are grateful to Arend Kolk (Royal Tropical Institute, Amsterdam, The Netherlands) for providing MAbs F30-5 and F183-24. Without the generosity of Theo Geijtenbeek, Yvette van Kooyk, and Jeroen den Dunnen (Molecular Cell Biology and Immunology, VU University Medical Center, Amsterdam, The Netherlands) in providing the DC-SIGN-Fc construct, Raji (+ DC-SIGN) cells, and technical assistance, this study would not have been possible. We appreciate very much the cooperation of Peter van der Ley (Netherlands Vaccine Institute, Bilthoven, The Netherlands) on this project. We thank Arun Mishra (School of Biosciences, University of Birmingham) and Marion Sparrius (Medical Microbiology, VU University Medical Center, Amsterdam, The Netherlands) for technical assistance and Wilbert Bitter (Medical Microbiology, VU University Medical Center, Amsterdam, The Netherlands) for helpful advice.

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Editor: J. L. Flynn